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Tel 415 576-0200

Seattle, Washington
Tel 206 467-9600

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Palo Alto
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Patrick A. Schneider et al.

Application No.: 09/485,193

Filed: June 3, 1999

For: USE OF PROTHYMOSIN IN THE DIAGNOSIS AND TREATMENT OF ENDOMETRIOSIS

Examiner: Stephanie W. Zitomer

Art Unit: 1634

COMMUNICATION

Assistant Commissioner for Patents
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Sir:

In response to the Examiner's telephonic message of March 3, 2003, Applicant provides herewith a complete copy of

Sburlati et al., Proc. Natl. Acad. Sci. 88:253-257 (1991).

Respectfully submitted,

Nathan Cassell

Nathan S. Cassell
Reg. No. 42,396

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, 8th Floor
San Francisco, California 94111-3834
Tel: 650-326-2400
Fax: 415-576-0300
PA 3288468 v1

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Cell Biology

Prothymosin α antisense oligomers inhibit myeloma cell division

(oligodeoxyribonucleotide/synchronized cells/hybrid arrest of translation/intracellular oligonucleotide stability)

ADRIANA R. SBURLATI, RICHARD E. MANROW, AND SHELBY L. BERGER*

Section on Genes and Gene Products, Laboratory of Biochemistry, National Cancer Institute, Bethesda, MD 20892

Communicated by Gilbert Ashwell, October 8, 1990 (received for review August 6, 1990)

ABSTRACT The function of prothymosin α has been investigated by using four different antisense oligodeoxyribonucleotides directed at selected regions of its mRNA. In every case, when synchronized human myeloma cells were released from stationary phase by incubation in fresh medium containing antisense oligomers, cell division was prevented or inhibited; sense oligomers and random antisense oligomers had no effect. A detailed analysis of synchronized cell populations indicated that sense-treated and untreated cells divided ≈17 hr after growth initiation, whereas cells incubated with antisense oligomer 183, a 16-mer targeted 5 bases downstream of the initiation codon, entered mitosis approximately one cell division late. The failure to divide correlated directly with a deficit in prothymosin α and with the continued presence of intact intracellular antisense oligomers over a period of at least 24 hr. Because antisense oligomers had no effect either on the timing of the induction of prothymosin α mRNA upon growth stimulation or on mRNA levels seen throughout the cell cycle, we concluded that antisense DNA caused specific hybrid arrest of translation. Our data suggest that prothymosin α is required for cell division. However, there is no evidence that prothymosin α directly regulates mitosis.

The function of prothymosin α is debatable. Evidence from this and other laboratories suggests that the protein is neither a precursor of thymosin α_1, a putative thymic hormone, nor a secreted thymic hormone itself (1–3). Instead, several observations support a role in cell proliferation: (*i*) Prothymosin α mRNA and protein are present in virtually all mammalian tissues, and a homologous protein has been detected in yeast (1, 4–8); these findings are consistent with an activity essential to most cells. (*ii*) The amounts of prothymosin α and its mRNA are roughly proportional to the proliferative activity of the tissue from which they are isolated (1, 4, 7). (*iii*) Prothymosin α mRNA is induced in normal human lymphocytes and in serum-starved NIH 3T3 cells upon growth stimulation with mitogens or serum, respectively (1).

Using COS cells transfected with the human prothymosin α gene, we have recently shown that prothymosin α is a nuclear protein. We have also found that chimeric proteins composed of all or part of prothymosin α fused with β-galactosidase are targeted to the nucleus only when the basic amino acids at the carboxyl terminus of prothymosin α are included (9). The presence of a nuclear localization signal indicates a function carried out, at least in part, in the nucleus; the precise nature of that function is unknown.

In the present study, we have directly evaluated the relationship between prothymosin α and cell division. Using antisense oligomers to inhibit accumulation of prothymosin α in a synchronized population of myeloma cells, we have established that cells deficient in prothymosin α cannot divide, that the inhibition is reversible, and that degradation of intracellular antisense oligomers accompanies resumption of the cell cycle.

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MATERIALS AND METHODS

Myeloma Cells. Type RPMI 8226 human myeloma cells were cultured as described (9). Cells were synchronized by incubation for 5–7 days at 2–5 × 10^6 cells per ml without change of medium and suspended in fresh medium at the same concentration to initiate growth, with oligomers and tracer as noted. In selected experiments, cells were diluted 10-fold.

Oligomers. Antisense and sense oligodeoxyribonucleotides, with base sequences complementary and identical, respectively, to those of prothymosin α mRNA, were purchased in ≈1000 OD amounts from Synthecell (Rockville, MD). Terminal phosphates were absent. The names and sequences of the four antisense oligomers used in this study are as follows: 178, 5′-GTCTACGGCTGCGTCTGACAT-3′; 183, 5′-GGTGTCTACGGCTGCG-3′; 448, 5′-CATCATCT-TCAGCTGC-3′; and 1019, 5′-CCAACTACTTATAGTA-CAG-3′. Complementary sense counterparts bear the same names. Except where noted, oligomer concentrations were 40 μM. The quality of the oligomers was evaluated by visual inspection of 20% polyacrylamide gels; greater than 90% of the ethidium bromide-stained DNA was the correct size relative to a standard 16-mer.

Radioactive oligomers (600 ng) were synthesized from oligomers lacking the 3′-terminal nucleotide specified above by extending them to full length with the appropriate deoxyribonucleoside [^{32}P]triphosphate (3000–6000 Ci/mmol, Amersham; 1 Ci = 37 GBq) in 20 μl of tailing buffer (BRL) containing bovine serum albumin at 100 μg/ml, 40 μCi of label, and 150 units of terminal deoxynucleotidyltransferase (Boehringer Mannheim) at room temperature for 5 min (10). Oligomers were also labeled for 30 min at 37°C in half the above volume with 50 units of terminal transferase and 50 μCi of [^{3}H]triphosphate (≈50–70 Ci/mmol). DNA was purified by extraction with phenol/chloroform/isoamyl alcohol, 25:24:1, boiled, subjected to G-25 Sephadex spun column fractionation, and evaluated electrophoretically in 8% polyacrylamide sequencing gels in the presence of 8 M urea (2); markers included $(dT)_8$ and $(dT)_{12-18}$. Radioactivity incorporated into DNA was quantified by binding to DE-81 filters (11).

Oligomer stability was determined in 1-ml cultures with 40 μM [^{32}P]DNA (2 × 10^6 cpm/ml). Cells were washed extensively with unlabeled ice-cold phosphate-buffered saline (PBS) and lysed in 0.5 ml of low-salt Tris I/lysing buffer I, 3:1 (12); nuclei resuspended in 500 μl of PBS, cytoplasm (500 μl), and 2 μl of the cell-free culture fluids in 500 μl of PBS were incubated overnight at 65°C with 100 μg of proteinase K and 5 μl of 10% SDS. In each case, the size of the aliquot examined electrophoretically was based on the recovery of

*To whom reprint requests should be addressed at: National Institutes of Health, Building 10, Room B1B38, Bethesda, MD 20892.

an external standard (1.2×10^6 cpm of an otherwise identical ^{3}H-labeled oligomer).

Assay of Prothymosin α. The amount of prothymosin α produced during the cell cycle was determined by using 2.5×10^6 oligomer-treated cells labeled with 250 μCi of [^{3}H]-glutamic acid in 1-ml cultures from zero time until time of harvest. Washed cells were lysed by boiling in 50 μl of buffer (10 mM Tris·HCl, pH 7.5/0.5% SDS/0.5% 2-mercaptoethanol/pepstatin at 42 μg/ml/phenylmethylsulfonyl fluoride at 0.52 mg/ml/leupeptin at 1.5 μg/ml. A synthetic external standard protein (25,000 cpm) derived from a prothymosin α pseudogene (gene 112) synthetic mRNA was also added to each sample without regard for cell count. Our methods for generating synthetic proteins are detailed elsewhere (9). Acetone-precipitated proteins (13) were analyzed by using nonequilibrium two-dimensional gel electrophoresis (9); protein spots were quantified with a Molecular Dynamics 300A computing densitometer to scan gel autoradiographs.

RNA Transfer Blots. RNA was isolated, fractionated electrophoretically, transferred to Nytran membranes (Schleicher & Schuell) and probed with prothymosin α cDNA and with a class I major histocompatibility gene, essentially as noted earlier (1).

RESULTS

Effect of Antisense Oligomers on Cell Division. Experiments with antisense oligomers to prothymosin α were undertaken to explore the apparent role of the protein in cell proliferation. Our goal was to make cells deficient in prothymosin α and to determine the effect on growth and division of a synchronized population of myeloma cells. Toward this end, four antisense oligomers were synthesized; two of them, 178 and 183, are directed against sequences coding for the amino-terminal end of prothymosin α; one oligomer, 448, is aimed at nucleic acid sequences upstream of the stop codon, whereas antisense oligomer 1019 can hybridize with the 3′-noncoding region of prothymosin α mRNA. The positions of the oligomers with respect to prothymosin α mRNA are illustrated in Fig. 1. Effect of the oligomers on cell division was assessed as follows: Myeloma cells were grown to stationary phase; at time 0 they were suspended at the same concentration in fresh medium containing antisense or sense oligomers or in medium without additions. Thereafter, cells were counted at daily intervals. As shown in Table 1, cells incubated without oligomers or with any of the four sense oligomers doubled in number after 24 hr and, except for sense oligomer 178 where data are lacking, doubled in number again after 48 hr. In contrast, cells treated with any of the four antisense oligomers did not divide within 24 hr, or experienced significant inhibition of proliferation. However, in the interval between 24 and 48 hr, all groups of antisense-treated cells recovered and divided, resulting in cell numbers which were about half those of sense-treated samples or untreated controls measured on the second day. The antisense 183

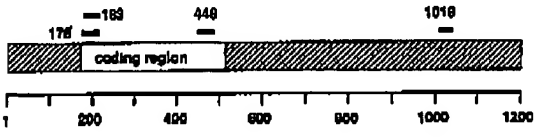

FIG. 1. Map of prothymosin α mRNA indicating regions targeted by oligomers. Antisense oligodeoxyribonucleotides are named for the 5′-most base of prothymosin α mRNA to which they can hybridize; sense oligomers are named similarly, except that they are identical to mRNA. The initiation codon is found at position 178, and the stop codon is at position 511. Hatched regions are noncoding; the prothymosin α coding region is noted. Oligomers are not drawn to scale.

oligomer produced the most dramatic and reproducible results, whereas cell division always occurred to some extent with antisense oligomer 1019.

Two further controls were performed. (*i*) The effect of antisense 183 and sense 183 oligomers was compared with that of random antisense and sense 183 oligomers that were constructed by reversing the sequences from 5′ to 3′. Thus, random antisense 183 is 5′-GCGTCGGCATCTGTGG-3′. In these experiments, sense, random sense, and random antisense DNA had no effect on cell division, whereas the standard antisense oligomer inhibited cell division as detailed above (Table 1). These findings indicate that the antiproliferative effect of antisense oligomers to prothymosin α is a function of the correct sequence and not of gross oligomer composition. The effect of an antisense oligomer directed at β_2-microglobulin, 5′-GCTAAGGCCACGGAGCG-3′, was tested at a concentration of 40 μM. At time 0, controls lacking oligomers and antisense-treated samples contained 4.7×10^6 cells; 24 hr later both samples contained 9.9×10^6 cells. These results rule out the possibility that antisense oligomers, in general, inhibit cell growth by some form of nonspecific toxicity.

The 183 oligomer pair was chosen for further evaluation. Oligomer concentrations from 10 to 80 μM were tested under conditions identical to those described above. At 10 μM, antisense 183 had no effect on cell division; at 20 μM, the effect was modest, resulting in a ratio of sense- to antisense-treated cells of 1.3 in 24 hr, and at either 40 μM (Table 1) or 80 μM the effect was maximal with the aforementioned ratio equal to 2. Furthermore, the ability of antisense 183 oligomers to prevent growth was observed in rapidly growing cells, as well as in cells released from stationary phase by incubation in fresh medium at a cell concentration 10-fold lower than that specified in Table 1 (data not shown). Apparently, the effect of prothymosin α antisense DNA on cell growth was independent of culture conditions.

The experiments described above establish the fact that antisense oligomers to prothymosin α mRNA inhibit cell division. However, they do not reveal the precise time interval between the division of control cells and that of cells in the process of overcoming antisense-induced repression of growth. This issue is addressed in Fig. 2, where cell numbers were monitored throughout two normal cell cycles. As in Table 1, cells were grown to stationary phase and subsequently transferred to fresh medium with and without 183 oligomers. Inspection of the data revealed that both sense-treated cells and untreated controls divided virtually synchronously at ≈16–18 hr and again at ≈38 hr. The antisense-treated cells did not divide at 16 hr; instead, they divided virtually synchronously at ≈36 hr or approximately one cell division late. Fig. 2 *Inset*, which is derived from a different experiment, illustrates both the synchrony of division of antisense-treated cells and the daily variation observed in cell-cycle kinetics. These results suggest that the deficiency of prothymosin α prevented the cells from dividing on schedule.

Prothymosin α levels were subsequently determined by using an assay based on two-dimensional gels. This method was developed in response to the lack of an antibody with sufficient titer or specificity to quantify prothymosin α (7, 9, 14). Hence, an experiment identical to that in Fig. 2 was performed, except that [^{3}H]glutamic acid was added along with the oligomers at time 0. Labeled proteins were examined electrophoretically (Fig. 3) at intervals thereafter and quantified relative to a radioactive external standard derived from a prothymosin α pseudogene (see *Materials and Methods*). This approach does not permit an assessment of total prothymosin α remaining in the cells, but it does allow a comparison to be made between the relative amounts of labeled prothymosin α in the antisense- and sense-treated

Table 1. Effect of oligomers on growth of synchronized human myeloma cells

	Effect of oligomers on cell growth, $\times 10^{-7}$ cells/ml													
	Exp. 1									Exp. 2				
		178		183		448		1019			183		Random*	
Time, hr	None	A	S	A	S	A	S	A	S	None	A	S	A	S
0	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.48	0.48	0.48	0.48	0.48
24	0.67	0.42	0.62	0.25	0.76	0.34	0.70	0.37	0.78	1.25	0.52	1.10	1.13	1.37
48	1.29			0.68	1.26	0.69	1.42	1.07	1.49					

Myeloma cells were grown to stationary phase, suspended in fresh medium with or without 40 μM of designated oligomer, and divided into separate 1-ml cultures. Number of cells was evaluated at time 0, 24 hr, and 48 hr. A, antisense oligomer; S, sense oligomer; 178, 183, 448, and 1019, oligomers targeted to four different regions of prothymosin α mRNA (see Fig. 1).
*Refers to the random 183 oligomers constructed by synthesizing both antisense and sense 183 oligomers backwards.

populations. In this system, prothymosin α (Fig. 3, arrow) is easily recognized by its pI value of 3.55 and its molecular mass of 13 kDa. The identification of prothymosin α in two-dimensional gels has been verified by demonstrating comigration of labeled synthetic and natural prothymosin α and by comparing patterns obtained from COS cells, either untransfected or transfected with the prothymosin α gene (9).

Fig. 3 shows that 18 hr after growth stimulation prothymosin α was clearly deficient in antisense-treated cells. The sense-treated population contained ≈4-fold more prothymosin α than the antisense-treated cells with respect to either the external standard or the label incorporated into other proteins viewed in the same gel. Before 18 hr, measurements were difficult owing to poor incorporation of radioactive precursors into protein; nevertheless, a difference of ≈2-fold, favoring sense-treated cells, could be ascertained, starting at ≈10 hr. By 24 hr the ratio of prothymosin α in sense-to-antisense-treated cells reflected the partial recovery of the latter population; again, a 2-fold difference was obtained. It should be noted that approximately equal radioactivity was loaded on the gels to obviate the effect of cell division in the sense-treated population. The extended labeling periods required to acquire sufficient radioactivity for protein quantification probably result in [^{3}H]prothymosin α levels which approach total accumulation; our assay is not designed to measure pulse-labeled or newly synthesized molecules. The data indicate that treatment of myeloma cells with antisense 183 oligomers specifically reduces the amount of prothymosin α.

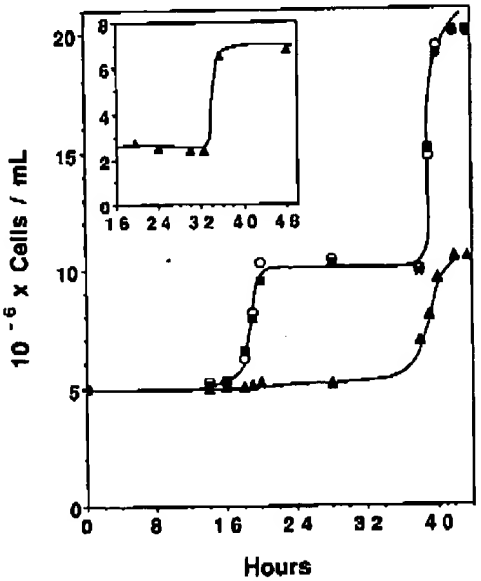

FIG. 2. Effect of 183 oligomers on synchronized human myeloma cells. Cells were grown to stationary phase, suspended in fresh medium with or without 40 μM 183 oligomers, and divided into separate 1-ml cultures. At stated times they were counted. Two independent flasks of cells were used at each time; hence each point is an average. ■, Sense oligomer; ▲, antisense oligomer; and ○, untreated control. (*Inset*) Part of cell cycle in a similar experiment done with the same antisense oligomer.

Effect of Antisense Oligomers on mRNA. Antisense DNA oligomers are believed to function in cells in one of two ways; either the DNA hybridized to mRNA generates a substrate for ribonuclease H, an enzyme capable of degrading the RNA moiety of such heteroduplexes, or the presence of bound DNA on mRNA causes hybrid arrest of translation (15, 16). In the former case, an effect of prothymosin α antisense DNA at the level of mRNA would be expected. In an attempt to resolve the issue, cytoplasmic RNA was isolated from antisense- and sense-treated cells which had been released from stationary phase, as described above. Blots derived from these samples are displayed in Fig. 4. Regardless of which 183 oligomer was included in the cell suspension, it is clear that prothymosin α mRNA was induced ≈4–6 hr after the initiation of growth. Untreated controls exhibited identical induction kinetics (data not shown). It can also be seen that,

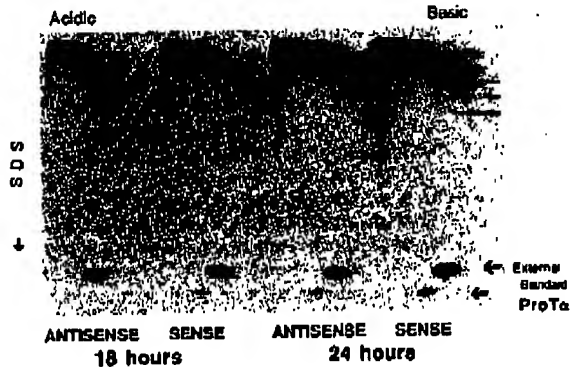

FIG. 3. Effect of 183 oligomers on prothymosin α accumulation in synchronized human myeloma cells. Cells were grown to stationary phase and suspended in separate 1-ml cultures containing 2.5 × 10^6 cells and 250 μCi of [^{3}H]glutamic acid in fresh medium with 40 μM of either sense or antisense 183 DNA. Cells were harvested after the stated period, lysed with 25,000 cpm of an external standard, and boiled in SDS. Lysates were homogenized by multiple passages through a 27-gauge needle, acetone-precipitated, and resuspended in sample buffer. Prothymosin α was quantified in two-dimensional gels in which the horizontal dimension was nonequilibrium isoelectric focusing, and the vertical dimension was SDS/15% PAGE. Only the most acidic 3 cm of each isoelectric focusing gel (pH ≈ 3.5–4) were loaded on the second-dimensional gel. Approximately 20% of each sample was analyzed. Arrows, external standard and prothymosin α (proT α).

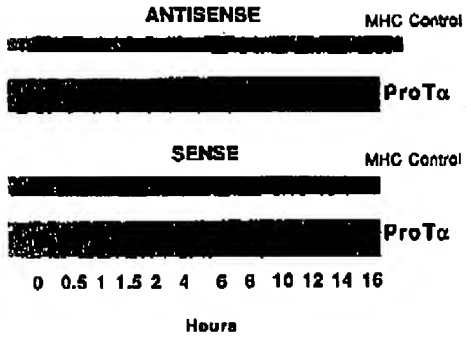

FIG. 4. Analysis of prothymosin α mRNA in synchronized human myeloma cells. Stationary-phase cells were resuspended in fresh medium at 3×10^6 cells per ml with 40 μM sense or antisense oligomers. At the stated time cytoplasmic RNA was obtained, fractionated electrophoretically in 1.2% agarose formaldehyde gels run in 3-(4-morpholino)propanesulfonic acid buffer made 1.1 M in formaldehyde, blotted onto Nytran (Schleicher & Schuell), and hybridized with labeled prothymosin α cDNA (proT α) and a class I major histocompatibility complex (MHC) probe. Each lane contains ≈2.5 μg of RNA.

relative to a major histocompatibility complex mRNA control, the levels of prothymosin α mRNA at each time point were identical regardless of the oligomer used. These results were extended to the 178 and 1019 oligomer pairs (data not shown). It seems clear that prothymosin α mRNA was not specifically degraded. Therefore, prothymosin α antisense oligomers seem to have prevented or inhibited translation by specific hybridization to otherwise translatable mRNA.

Stability of Sense and Antisense Oligomers. The effect of antisense oligomers on proliferation of myeloma cells lends itself to a simple interpretation: Oligomers in the culture fluids are taken up by cells but have no effect on the induction of mRNA which occurs 4–6 hr after release from stationary phase. The oligomers must accumulate intracellularly at a concentration sufficient to inhibit translation of prothymosin α mRNA for at least 18 hr. At ≈24 hr the intracellular oligomer concentration must decline in order to account for the observed increase in prothymosin α between 18 and 24 hr and the eventual division of the antisense-treated population. As a test of these hypotheses, it was necessary to monitor uptake and stability of sense and antisense oligomers. For this purpose, labeled oligomers were required; their synthesis was accomplished by the controlled addition of the appropriate radioactive nucleotide to truncated 183 oligomers missing the 3′-terminal residue. The result was a radioactive molecule, identical to those used throughout this study but with a [^{32}P]phosphodiester bond at the 3′ end. Because oligomers were to be recovered from fractionated cells, we also synthesized ^{3}H-labeled oligomers, labeled similarly for use as external standards. Oligomer stabilities were evaluated in sequencing gels, autoradiographs of which are presented in Fig. 5. In the medium, both sense and antisense oligomers decayed similarly with half-lives of ≈1 hr based on binding of radioactive molecules to DE-81 filters (data not shown). However, analysis in gels revealed two additional facts about the extracellular oligomers: (*i*) there was microheterogeneity including failure sequences, particularly in the antisense population; and (*ii*) decay was not first order since a significant quantity of intact molecules remained at 24 hr in each case. Intracellular oligomer stabilities differed markedly. The sense oligomers were so unstable that their presence was difficult to discern even at time 0. Within these cells, [^{32}P]DNA capable of binding to DE-81 filters was found from 0–6 hr (data not shown); i.e., rapid intracellular breakdown of intact oligomers could not be distinguished from uptake of fragments. In comparison, the antisense oligomers entered the cells as intact entities, remained stable for at least 6 hr, decayed significantly by 24 hr, and disappeared by 48 hr. Furthermore, the nucleus appeared to act as reservoir for antisense DNA, with levels at 6 hr routinely exceeding those in the cytoplasm. The data are consistent with a model in which binding of antisense oligomers to prothymosin α mRNA or premRNA resulted not only in increased oligomer stability inside the cell but also in hybrid arrest of translation of mature mRNA molecules. The data also support the temporal aspects of prothymosin α antisense inhibition of cell proliferation suggested above.

DISCUSSION

Antisense RNA and synthetic antisense DNA oligomers inhibit gene expression in eukaryotic cells by selectively interfering with the translation or the stability of target mRNAs (15, 16). In the present study, we have used normal DNA molecules, without terminal phosphates, to investigate the function of prothymosin α. When human myeloma cells were incubated with any of four antisense oligodeoxyribonucleotides, cell division was prevented or inhibited. Two of the oligomers, 178 and 183, were directed at nucleic acid sequences coding for the amino terminus of the protein; one oligomer, 448, was aimed at sequences ≈70 bases upstream of the stop codon; and one oligomer, 1019, could hybridize deep in the 3′-noncoding region of prothymosin α mRNA. Because all of the antisense oligomers, regardless of their targets, were inhibitory, whereas their sense counterparts and a random antisense oligomer were not, the effect on cell division appears to be specific. Evidently, the transient deficit of prothymosin α postpones mitosis.

The mechanism by which the oligomers modulate cellular processes was also examined. The amount of cytoplasmic prothymosin α mRNA found in cells as a function of the cell cycle was identical in the presence of either sense or antisense oligomers, or in their absence. Furthermore, the timing of prothymosin α mRNA induction upon growth stimulation remained the same whether cells were treated

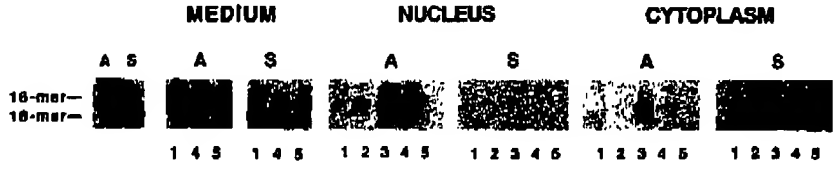

FIG. 5. Analysis of the stability of 183 sense and antisense oligomers in the medium, cytoplasm, and nuclei of myeloma cells. Stationary-phase cells were resuspended in fresh medium containing either ^{32}P-labeled 183 antisense or sense oligomers at 40 μM (2×10^6 cpm/ml) and divided into individual 1-ml cultures. At stated time, cells were harvested and lysed. The ^{3}H-labeled external standard oligomer was added to separated cytoplasmic and nuclear fractions before subsequent processing. Oligomers were quantified in 8% polyacrylamide-urea sequencing gels. Autoradiographs of fixed dried gels display ^{32}P-labeled oligomers exclusively. A, antisense; S, sense. Lanes 1–5 refer to materials obtained at time 0, 1 hr, 6 hr, 24 hr, and 48 hr, respectively. Labeled oligomers that had not been exposed to cells are at left; the position of the antisense (*Upper*) and sense (*Lower*) 16-mer is indicated.

with DNA of any sort or reserved as untreated controls. Therefore, these antisense oligomers appear to cause hybrid arrest of translation. The antisense-induced deficiency in prothymosin α, relative to other acidic proteins viewed in two-dimensional gels, corroborates this conclusion. The results also suggest that sequences at the 3′ ends of mRNA are important for translation (17), an observation made previously with antisense RNA transcripts complementary to the entire 3′-noncoding region of creatine kinase mRNA (18).

The uptake and stability of the oligomers were measured by using [^{32}P]DNA molecules. Our data show that antisense oligomers were taken up by myeloma cells almost immediately and that they remained detectible, intracellularly, for at least 24 hr. These results are consistent with the observed reversible effect of prothymosin α antisense on cell division. We also noted that antisense oligomers were concentrated in the nucleus. Antisense, but not sense, oligomers seem to be protected, probably because they hybridize to RNA transcripts and remain bound during transport into the cytoplasm. These insights were obtained with the use of native ^{32}P-labeled oligomers that were labeled internally at the 3′-most phosphodiester bond. Molecules modified with radioactive 5′-end phosphates (19) or bulky groups (20, 21) have been used by others, resulting in conclusions regarding the behavior of oligomers quite different from ours. It should be emphasized, in this connection, that reisolation of intact molecules appears the only reliable method for assessing oligomer survival.

An analysis of the fluctuation of prothymosin α throughout the cell cycle provides clues to its function. When stationary-phase myeloma cells are exposed to fresh medium, prothymosin α mRNA is induced after ≈6 hr, with mitosis occurring at ≈17 hr. From these data it might be inferred that prothymosin α levels are not critical for the transition from G_0–G_1 phase but become important later in the cell cycle. Sustained high levels of prothymosin α mRNA during S phase at 10–12 hr (A.R.S., unpublished work) through G_2 phase support this view. By considering the results with antisense DNA, one can further narrow the interval during which prothymosin α appears to be required. Despite early uptake of oligomers, cells do not become depleted in prothymosin α until ≈10 hr after growth stimulation; lowest levels occur at 18 hr. This observation implies that prothymosin α functions in G_2 phase, in preparation for entry into M phase. Nevertheless, we have no evidence that prothymosin α directly regulates cell division. Treatment with prothymosin α antisense oligomers also results in a small (10%) reduction in total protein synthesis (A.R.S., unpublished work); consequently, an effect on mitosis mediated through a deficiency in one or more other proteins must be considered.

Two possible roles for prothymosin α can be hypothesized. Since the protein is highly acidic and localized to the nucleus, it could function as a secondary transcription factor, perhaps similar in some respects to the VP16 protein of herpes viruses (22). Because the accumulation of many proteins is unchanged in the presence of our antisense DNA, prothymosin α probably does not affect transcription globally. Accordingly, it could be a transcription factor that regulates a limited array of genes, some of which affect cell division. An alternative position casts prothymosin α in the role of a shuttle protein conveying a non-targeted molecule essential for cell growth and division to its nuclear site of action. In this way, it might resemble adenovirus preterminal protein (23). Prothymosin α not only meets the physical requirements of this hypothesis but, because it interacts weakly with nondiffusible elements in cell nuclei (9), also satisfies the criteria from a functional point of view as well.

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